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VASODILATOR EFFECTS OF SARAFOTOXINS AND ENDOTHELIN-1 IN SPONTANEOUSLY HYPERTENSIVE RATS AND RAT ISOLATED PERFUSED MESENTERY

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Abstract—Sarafotoxins (SRTa, SRTb and SRTc) and ET-1 produced a potent vasodilator effect in spontaneously hypertensive rats *in vivo* and in rat isolated perfused mesenteries *in vitro*. Among these peptides SRTc demonstrated the most potent vasodilator activity, and was three times more active than SRTa in both preparations. These peptides induced endothelium-dependent vasodilatation *in vitro* and pretreatment with methylene blue inhibited this effect, while exposure to the antagonists of other vasodilators did not. In contrast, [nitrophenylsulfenylated Trp^{21}]SRTc, SRTc(1-18) and reduced and *S*-carboxymethylated SRTc caused no vasodilatation in either animal model; the vasodilator effect of acetylated SRTc was less potent than that of SRTc. These results suggest that (i) the vasodilatations of these peptides may be exerted through the release of endothelium derived relaxing factor; (ii) the C-terminal Trp^{21} and disulfide bonds are essential; and (iii) the N-terminal amino group plays an important role in vasodilator activity.

Sarafotoxin S6a (SRTa), sarafotoxin S6b (SRTb) and sarafotoxin S6c (SRTc) are new cardiotoxic peptides isolated from the venom of the burrowing asp, *Atractaspis engaddensis* [1, 2]. The amino acid sequence of these peptides shows an extensive homology with that of endothelin-1 (ET-1) [3, 4], a new 21-residue vasoconstrictor peptide obtained from porcine endothelial cells [5]. We have previously demonstrated that sarafotoxins produce a vasoconstrictor effect in rat thoracic aortas, rat isolated perfused mesenteries and pithed rats, and that the rank order of potency is SRTb > SRTa > SRTc [6]. Moreover, it has been shown that ET-1 has a vasodilator effect on rat mesenteric circulation through the release of endothelium derived relaxing factor (EDRF) [7]. In the present study, the vasodilator activity of sarafotoxins was studied in spontaneously hypertensive rat (SHR) and rat isolated perfused mesentery. Furthermore, a structure–activity study on sarafotoxins using a variety of synthetic SRTc analogs was performed in these animal models.

MATERIALS AND METHODS

Depressor activity in SHR. Under light ether anesthesia, two catheters were inserted into each male spontaneously hypertensive rat (SHR, Charles River, Japan; 14 weeks old, 300–350 g), one into the left femoral artery to measure blood pressure, and the other into the caudal vein to administer test drugs. The animals were suspended horizontally in a cloth tube with slits through which the four limbs, tail and catheters were exposed, and the experiments were initiated after the animals had regained consciousness. Mean arterial blood pressure (MAP) was measured by a pressure transducer (MPU-0.5, Tohyo and 1258, NEC San-ei) and heart rate was recorded by means of a systolic pressure-triggered tachometer (1321, NEC San-ei). Electrocardiograms (ECG) were recorded through a lead II connection using an oscilloscope (MS6, Medelec). Under these conditions, MAP was 176.0 ± 0.9 ($N = 49$, mean ± SE). The peptides were injected into the caudal vein at a fixed volume of 1 mL/kg.

Rat isolated perfused mesentery preparation. The mesenteric arteries of male Sprague–Dawley rats (Charles River, Japan; 300–350 g) were isolated and perfused as described by McGregor [7]. The rats were anesthetized with sodium pentobarbital (35 mg/kg i.p.) and treated with heparin (1300 units/kg i.v.). The superior mesenteric artery was cannulated at its origin, flushed with 15 mL of Krebs–Ringer solution and isolated by cutting along the intestinal border of the mesentery. Mesenteric vasculature preparations were perfused with Krebs–Ringer solution using a perista pump (SJ-1215, Atto) at a rate of 5 mL/min. The Krebs–Ringer solution [(mM): NaCl 130, KCl 4.8, $CaCl_2$ 2.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, dextrose 5.5], was maintained at 37° and continuously bubbled with a mixture of 95% O_2 and 5% CO_2. The isolated mesenteric artery was initially perfused for 30 min as a conditioning period. The perfusion pressure was measured by means of a pressure transducer (MPU-0.5, Tohyo and 1258, NEC San-ei) and the peptides were injected into the arterial cannula using a microsyringe (Kloehn; volumes of 3–30 μL). Vasodilator responses to the peptides were recorded in mesenteric beds preconstricted with phenylephrine (30 μM, perfusion pressure 40–80 mmHg, $N > 50$), and were expressed as percentages of the vasodilatation induced by carbachol (60 pmol) in each preparation.

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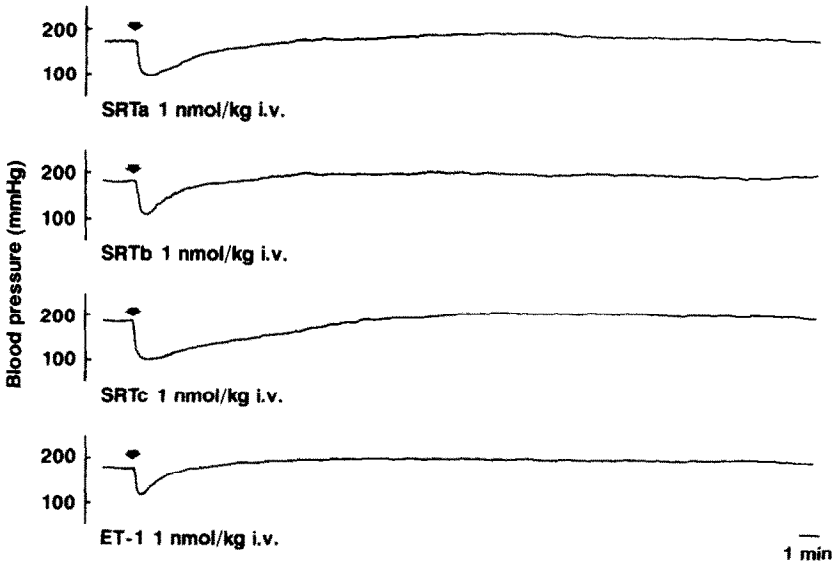

Fig. 1. Typical recordings of depressor responses of spontaneously hypertensive rats to intravenous bolus injections of sarafotoxins and ET-1.

Drugs. SRTa, SRTb and SRTc were isolated and purified from the venom of *Atractaspis engaddensis*, as described previously [3]. The venom was provided by Dr E. Kochva (Tel Aviv University). SRTc(1-18) was prepared by digesting SRTc with carboxypeptidase Y (Oriental Yeast Co). [Homoserine^6]-SRTc ([Hse^6]SRTc) was converted from SRTc by treatment with cyanogen bromide using 100 mol eq. of BrCN in 70% formic acid at 37° for 6 hr. Under the above conditions, the methionine residue at position 6 of SRTc was converted to a homoserine residue but the resulting Hse^6-Thr^7 bond did not cleave. These peptides were purified by means of reverse-phase HPLC using a Pep RPC HR5/5 column (Pharmacia). Reduced and *S*-carboxymethylated (RCM), acetylated (Ac) or nitrophenylsulfenylated (NPS) products of SRTc were separated by reverse-phase HPLC. ET-1 was obtained from Peptide Institute (Osaka). Peptides were dissolved to 10^{-5} M in PBS containing 0.1% bovine serum albumin (Sigma) and stored in aliquots at −20° until use.

Statistical analysis. Data were expressed as means ± SE. Statistical analysis of data was conducted using the Student's *t*-test (statistical significance, $P < 0.05$).

RESULTS

When administered intravenously at a dose of 1 nmol/kg, SRTa, SRTb, SRTc and ET-1 caused similar biphasic changes in the MAP of SHR (Fig. 1). The initial response was characterized by significant and transient reduction in MAP, which peaked at about 1 min and subsided within 2 to 10 min. The initial response was followed by a secondary, slowly developing increase in MAP. In terms of heart rate, the MAP responses to sarafotoxins and ET-1 were accompanied by a transient tachycardia and, successively, with a mild but prolonged bradycardia. No changes other than heart rate were observed on ECG for either of the peptide injections. The maximum magnitude and 50% recovery time of the hypotensive response for SRTa (1 nmol/kg i.v.) were 71.5 ± 2.6 mmHg and 100.8 ± 5.0 sec (N = 4), respectively. The values were significantly larger and longer than those induced by the same dosage of ET-1 (56.0 ± 3.7 mmHg and 35.9 ± 2.1 sec, N = 4, respectively). The hypotensive effect of SRTb (a maximum decrease of 68.5 ± 1.4 mmHg and a 50% recovery time of 62.9 ± 1.3 sec, N = 4) was intermediate between those of SRTa and ET-1 at a dose of 1 nmol/kg. At the same dose, SRTc exhibited the most potent and long-lasting hypotensive response with a maximum decrease of 87.5 ± 1.9 mmHg and a 50% recovery time of 238.5 ± 8.5 sec (N = 4). Figure 2 shows the dose–response curves for the hypotensive effects of sarafotoxins and ET-1 in SHR. At doses ranging from 0.1 to 1.0 nmol/kg, all these peptides produced a dose-dependent hypotension; the effect was determined using the maximum decrease and the whole area MAP decrease. The rank order of potency for both parameters was SRTc > SRTa > SRTb > ET-1; the difference in the whole area decrease was more apparent than that seen in the maximum decrease.

In isolated perfused mesentery in which phenylephrine (30 μM) was infused to raise the perfusion pressure, SRTa and SRTc (100 pmol i.a.) produced a transient fall in perfusion pressure (Fig. 3). However, intraarterial injection of SRTb and ET-1 at a dose of 100 pmol elicited a biphasic response in perfusion pressure: a transient fall was followed by a protracted rise. Removal of endothelial cells with sodium deoxycholate (2.4 mM) treatment for 30 sec completely eliminated the relaxation response to SRTa and SRTc; instead, a mild increase in mesenteric arterial pressure was observed after administration of each peptide. The pressure response to

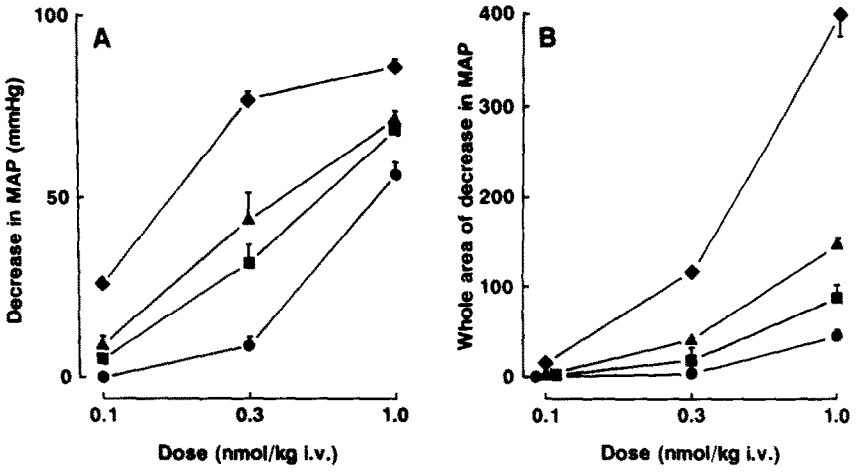

Fig. 2. Dose–response curves for the hypotensive effects of SRTa (▲), SRTb (■), SRTc (◆) and ET-1 (●) in spontaneously hypertensive rats. Hypotensive responses are expressed as a maximum decrease (A) and a whole area decrease in mean arterial blood pressure (MAP; B). Each point represents the mean ± SE (N = 4–5).

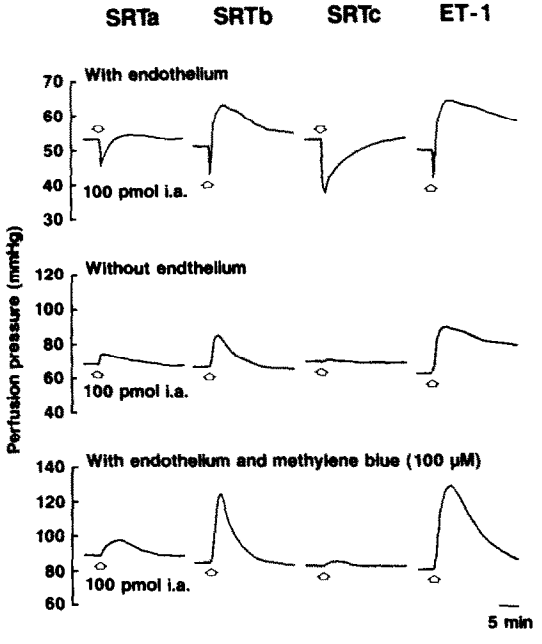

Fig. 3. Effects of sarafotoxins and ET-1 on perfusion pressure in rat isolated perfused mesenteries preconstricted with phenylephrine (30 μM), and the effects of removal of the endothelium and pretreatment with Methylene blue (100 μM).

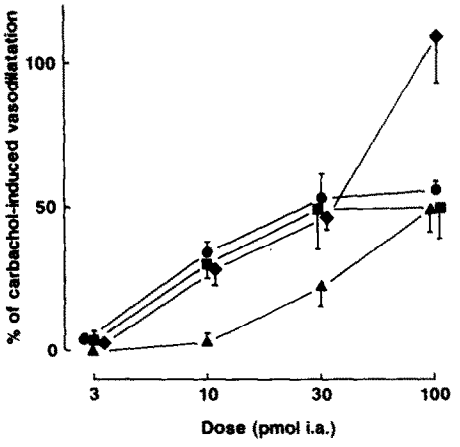

Fig. 4. Dose–response curves for the vasodilator effects of SRTa (▲), SRTb (■), SRTc (◆) and ET-1 (●) in rat isolated perfused mesenteries. Each point represents the mean ± SE (N = 3–5).

SRTb and ET-1 in the mesenteric artery treated with deoxycholate was a potent vasoconstriction without relaxation. While pretreatment with Methylene blue (100 μM) diminished the vasodilatations induced by these peptides, pretreatments with propranolol (10^{-6} M), atropine (10^{-6} M), pyrilamine (10^{-6} M), cimetidine (10^{-6} M) and indomethacin (5×10^{-6} M) were ineffective. Furthermore, prior administration of either sarafotoxins or ET-1 weakened the vasodilator effect. The dose–response curves for the vasodilator effects of sarafotoxins and ET-1 are shown in Fig. 4. SRTa and SRTc produced a dose-dependent vasodilatation at doses ranging from 3 to 100 pmol and from 10 to 100 pmol, respectively. The vasodilator activity of SRTa was three times less potent than that of SRTc. The dose–response curves for the vasodilator effects of SRTb and ET-1 were similar to that of SRTc at doses ranging from 3 to 30 pmol and the vasodilator activity of both peptides reached a plateau at a dose of 100 pmol.

The vasodilator effects of SRTc analogs in SHR and in rat isolated perfused mesentery are summarized in Fig. 5. In SHR, the maximum decrease induced by either [Hse^6]SRTc or Ac-SRTc at a dose

Sarafotoxins and Endothelin-1	Amino-acid sequences	Vasodilator activity (%) in vivo	in vitro
SRTa	C S C K D M T/S D K E C L N F C H Q D V I W	81.7	45.6
SRTb	C S C K D M T D K E C L Y F C H Q D V I W	78.3	45.5
SRTc	C T C N D M T D E E C L N F C H Q D V I W	100	100
ET-1	C S C S S L M D K E C V Y F C H L D I I W	64.0	51.3
SRTc analogs			
RCM-SRTc	C T C N D M T D E E C L N F C H Q D V I W (CH_2SCH_2COOH on C; CH_2SCH_2COOH on C)	0	0
NPS-SRTc	C T C N D M T D E E C L N F C H Q D V I W (2-nitrophenylsulfenyl on W)	0	0
Ac-SRTc	C T C N D M T D E E C L N F C H Q D V I W ($NHCOCH_3$)	22.9	3.8
[Hse^6]SRTc	C T C N D Hse T D E E C L N F C H Q D V I W	63.4	40.4
SRTc(1-18)	C T C N D M T D E E C L N F C H Q D	0	0

Fig. 5. Structure–activity relationships of sarafotoxin vasodilator effects in spontaneously hypertensive rats *in vivo* and in rat isolated perfused mesenteries *in vitro*. Vasodilator activities *in vivo* (the maximum decrease at a dose of 1 nmol/kg i.v.) and *in vitro* (100 pmol i.a.) are expressed, in each case, as the percentage when SRTc-induced vasodilatation was taken as 100%. (C) Cystine, (D) aspartic acid, (E) glutamic acid, (F) phenylalanine, (H) histidine, (Hse) homoserine, (I) isoleucine, (K) lysine, (L) leucine (M) methionine, (N) asparagine, (Q) glutamine, (S) serine, (T) threonine, (V) valine, (W) tryptophan, (Y) tyrosine.

of 1 nmol/kg was approximately two to four times less potent than that of SRTc. Intravenous injections of RCM-SRTc, NPS-SRTc and SRTc(1-18) did not affect the MAP of SHR. The vasodilator activity of SRTc analogs tested in rat perfused mesentery closely parallel that of SHR; compounds having no hypotensive effect in SHR exhibited no dilatation in the mesenteric vessels *in vitro*.

DISCUSSION

The present study demonstrates that sarafotoxins and ET-1 produced a potent vasodilator effect in SHR *in vivo* and in mesenteric artery *in vitro*. These findings substantiate the previous observations reported by de Nucci and co-workers [8, 9] and Eglen *et al.* [10]. Among these peptides, the vasodilator activity of SRTc was most potent and was three times stronger than that of SRTa. SRTb and ET-1 produced less remarkable hypotension *in vivo*, while *in vitro* these peptides were almost equipotent with SRTc, especially at lower doses. A previous report emphasized that SRTb and ET-1 caused a potent and protracted vasoconstriction in several animal models [6]. However, it was assumed that in these results the vasoconstrictor response was much more remarkable than the vasodilator effect, so that the weak and transient hypotension elicited by these peptides was probably overlooked. The vasodilatation observed *in vitro* was abolished by removing the endothelial cells with sodium deoxycholate. Pretreatment with Methylene blue, an inhibitor of soluble guanylate cyclase, eliminated endothelium-dependent relaxation in these peptides. However, such an effect was unrelated to either the β-adrenergic, muscarinic and H_1- and H_2-histaminergic systems or to prostaglandin synthesis, since no inhibition was elicited by pretreatment with the corresponding antagonists. These findings seem to suggest that the vasodilator response to sarafotoxins and ET-1 may be mediated by EDRF. We have previously reported that the lysine residue at position 9 is important for inducing the vasoconstrictor activity of sarafotoxins and ET-1 [6]. However, the replacement of Lys^9 with Glu did not eliminate vasodilator activity, suggesting that this residue may not play any important role in the vasodilator activity induced by these peptides. NPS-SRTc, SRTc(1-18) and RCM-SRTc caused no vasodilatation in either animal model. Apparently, the C-terminal Trp^{21} and two sets of intrachain disulfide bonds are essential for the expression of vasodilator as well as vasoconstrictor activity. Furthermore, the lower potency of Ac-SRTc indicates that the N-terminal amino group is definitely important in eliciting the vasodilator activity of sarafotoxins and ET-1.

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